Patent Office
Canberra

I, JONNE YABSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PQ 2757 for a patent by WOMEN'S AND CHILDREN'S HOSPITAL and LUMINIS PTY LTD as filed on 10 September 1999.

WITNESS my hand this
Fifteenth day of August 2002

JONNE YABSLEY
TEAM LEADER EXAMINATION
SUPPORT AND SALES

OUR REF: 1777

P/00/009
Regulation 3.2

AUSTRALIA
Patents Act 1990

PROVISIONAL SPECIFICATION FOR AN INVENTION ENTITLED

Invention Title:	RECOMBINANT BACTERIUM EXPRESSING AN OLIGOSACCHARIDE RECEPTOR MIMIC
Names of Applicants:	WOMEN'S AND CHILDREN'S HOSPITAL and LUMINIS PTY LTD
Address for Service:	A.P.T. Patent and Trade Mark Attorneys G.P.O. Box 772, Adelaide, S.A. 5001

The invention is described in the following statement :

This invention relates to a means for adsorbing toxins or pathogenic bacteria from a particular environment, and in one specific application addresses the impact of toxins or infectious enteric pathogenic microorganisms by competitive adsorption of either toxins or pathogenic microorganisms.

Surfaces of all cells express a complexity of oligosaccharides that provide a number of functions. A primary one of these functions is determining on their own or together with other molecules, interactions with other cells or molecules. The nature, linkage and conformation of sugar residues of the oligosaccharides, and in particular residues at or close to the non-reducing terminus of an oligosaccharide, determines whether the oligosaccharide will or will not participate in a particular receptor-ligand interaction.

The susceptibility of an animal to infection and the eventual physiological site affected by an infection is to a large extent determined by the expression on the cell surface of such oligosaccharide receptors. In the case of enteric infections, one primary prerequisite for pathogenesis is that the microorganism persists in the intestinal lumen of the host. Generally this requires some form of adherence to the luminal epithelium, otherwise the micro-organism is flushed from the gut. Additionally for a toxigenic organism, the toxin also needs to bind to the luminal epithelium and perhaps be absorbed systemically to be effective, otherwise, it too would be flushed from the gut.

Certain surface structures of pathogenic and other bacteria known as adhesins mediate adherence to luminal epithelial cells. A number of adhesins are known and organisms without adhesins are generally of low virulence. Adhesins are proteinaceous factors which promote the adherence of bacteria and viruses to cells of their hosts. Adhesins can be either fimbrial or filamentous in structure or they may be afimbrial. Adhesins associated with fimbriae may be associated with accessory proteins such as the tip at an extreme end of the fimbrial structure. Where the adhesin is the tip protein, this can be referred to as a lectin if it binds to a carbohydrate receptor. The receptor on the host cell has in some cases been determined and shown to be a carbohydrate such as an oligosaccharide associated with a glycolipid or a glycoprotein.

The best characterised system from a molecular and biological viewpoint, is the P-fimbriae (also called pili) produced by uropathogenic *Escherichia coli*. This tip adhesin binds the glycolipid Gb_3 (defined later) and the fimbrial subunits can be purified by affinity chromatography using a Gb_3 mimic. Another well characterised group of adhesins are

those associated with enterotoxigenic *E. coli* (ETEC) strains which infect pigs to cause scours. These are termed the K88 type and a number of variants are known, being K88ab, K88ac and K88ad. The adhesins associated with these fimbriae have been shown to have different receptor requirements, which includes the presence of both glycolipid and protein receptors. The carbohydrate requirement has been characterised in at least some of these, for example K88ad uses carbohydrates of the lactoneotetraose series of glycolipids.

A number of toxins have been identified and include, Shiga toxins (also referred to as Shiga-like toxins and verotoxins), toxins produced by various species of *Clostridia*, including tetanus toxin, botulinum toxin, and *C. difficile* toxins A and B, Staphylococcal enterotoxins, *Escherichia coli* heat labile and heat stable enterotoxins and cholera toxin. Without toxin activity the majority of otherwise enterotoxigenic bacteria would be less capable of causing disease.

The receptors for the majority of adhesins and toxins identified to date are carbohydrate in nature. For example, the glycolipid globotriaosyl ceramide (Gb_3) which has the structure Galα[1-4]Galβ[1-4]Glc-ceramide, is the preferred receptor for most members of the Shiga toxin family. Similarly, the ganglioside G_{M1} is the receptor for cholera toxin and *E. coli* heat labile enterotoxin type I. *C. difficile* toxin A binds to several host receptors, all of which have in common a Galβ[1-4]GlcNAc moiety. The neurotoxin produced by *C. botulinum* is also believed to be specific for a sialic acid containing glycoprotein or glycolipid present on neurons. The terminal Galα[1-4]Galβ moiety present on Gb_3 is the receptor for P pili, the major adhesin of uropathogenic *E. coli* strains. Similarly, asialo-G_{M1} is the receptor for adhesive pili (CFAs) of enterotoxigenic *E. coli* strains. The sialated gangliosides NeuGc-GM_3 and NeuNAc-GM_3 have also been identified as the target cell receptors for porcine rotavirus strains, and it is presumed that rotavirus strains causing disease in humans also bind specific oligosaccharide moeities present on cell surface glycolipids.

The elucidation of the nature of oligosaccharides acting as receptors for particular toxins and pathogenic microorganisms has opened up a promising avenue in the diagnosis and potential treatment or prevention of diseases caused by these agents.

The use of the sugar residues forming receptors for toxins or adhesins has been proposed as a means of specifically identifying the toxins or bacteria involved in an infection. For

example, the ganglioside receptor G_{M1} is used as a specific capturing agent in ELISA assays for the presence of cholera toxin.

It has also been proposed to use synthetically prepared oligosaccharides as a means of adsorbing toxins or the like from samples. A problem arises, however, in the delivery of these compounds, because oligosaccharides are difficult or expensive to synthesise chemically, the conformation may not be appropriate, and the oligosaccharide may preferably need to be presented in an immobilized (non-diffusible) form. Thus, there is a need to provide a mechanism for delivery and presentation of the oligosaccharide moiety in an appropriate conformation in the environment where the toxin or pathogenic organism is to be adsorbed (for example the gastrointestinal tract).

Examples of proposed uses of receptors for adsorbing toxins or pathogenic organisms out of a sample include Krivan *et al* in US patent 5696000 which discusses the pharmaceutical use of certain tetra- and tri- saccharide receptors coupled to a carrier such as a liposome to inhibit the adherence of micro-organisms to susceptible cells. A similar use for toxins such as Shiga toxin, can be seen in US specification 5849714 to Rafter *et al* which discloses the use of a synthetic construct of sugar residues making up the globotriose receptor, coupled by a linker to an inert support for use in treatment of bacterial dysentery.

SUMMARY OF THE INVENTION

This invention arises from the construction of recombinant delivery bacteria expressing a chimeric lipopolysaccharide structure, the terminal sugars of which constitute a Shiga toxin receptor mimic. These recombinant delivery bacteria are effective at protecting susceptible cells from attack by the Shiga toxin whose receptor they mimic. The use of a chimeric carbohydrate moiety such as lipopolysaccharide means that the endogenous transport machinery of the delivery bacterium is used to appropriately display the receptor mimic. The effectiveness of providing a receptor mimic on the surface of a recombinant delivery bacterium has ramifications in relation to a broader range of toxins than simply Shiga toxins; it also has ramifications for other toxins such as those discussed above whose action requires recognition of oligosaccharide receptors. Additionally the receptors for adhesins of certain pathogenic bacteria and viruses are structurally similar to receptors for certain toxins, and the provision of receptors for such adhesins on a bacterial surface is proposed to have a protective effect by interfering with the pathogen and/or its capacity to infect host cells. Moreover it is proposed that the chimeric carbohydrate still has a

protective effect if secreted or released into the environment, not being attached to the delivery bacterial surface.

In one broad form of a first aspect, the invention could be said to reside in a method of reducing the amount of a toxin or a pathogenic organism from an environment, comprising the steps of introducing at least an inoculum of a recombinant delivery bacterium into the environment, said recombinant bacterium carrying exogenous nucleic acid encoding one or more exogenous sugar transferases, said one or more exogenous sugar transferases being specific for transfer of one or more sugar residues represented progressively from a non reducing terminal end of a receptor of either the toxin or an adhesin of the pathogenic organism to thereby form a chimeric carbohydrate molecule with a receptor mimic, said delivery bacterium being chosen to express an acceptor molecule that the one or more exogenous sugar transferases transfer said one or more sugar residues onto, to form the chimeric carbohydrate molecule, the receptor mimic being exposed to the environment for contact with the toxin or adhesin and being either anchored to the outer surface of the bacterium, or secreted into the environment.

In one broad form of a second aspect the invention could be said to reside in a recombinant delivery bacterium for use in the first aspect of the invention.

In a broad form of a third aspect, the invention could be said to reside in a recombinant DNA molecule including a glycosyl transferase encoding region, encoding one or more glycosyl transferases, and a control region for regulation of the expression of the glycosyl transferases, said transferases being specific for transfer of two or more sugar residues represented progressively from a non reducing terminal end of a receptor of a toxin or an adhesin of a pathogenic organism.

The control region may be such as to allow for constitutive expression of the transferases. Alternatively more complex control mechanism may be allowed for, and such more complex control mechanism may provide for induction of expression, as well as other known forms of control.

In a broad form of a fourth aspect the invention could be said to reside in a chimeric carbohydrate molecule comprising an acceptor molecule, with one or more sugar residues added to the non-reducing end thereof to form the chimeric carbohydrate molecule carrying a receptor mimic of a toxin or an adhesin of a pathogenic organism.

The chimeric receptor may be chosen to bind toxins, adhesins of enteric bacterial or viral pathogens, or lectins associated with these organisms.

The toxins are preferably those made in the gut requiring transport across the luminal epithelium and might be selected from, but not limited to, a group comprising shiga toxins, clostridial toxins, cholera toxins, *E. coli* enterotoxins, and Staphylococcal enterotoxins.

The shiga toxins might be selected from the group comprising, Stx, Stx1, Stx2, Stx2c, Stx2d, and Stx2e. In this case where the shiga toxin is Stx, Stx1, Stx2, Stx2c, or Stx2d, the receptor mimic preferably is formed by a terminal sugar moiety of Galα[1-4]Galβ[1-4]Glc. The one or more transferases is either an α1-4 galactosyl transferase capable of forming an α1-4 bond with a galactose residue bonded by a β1-4 bond to a glucose on the acceptor molecule, or both an α1-4 galactosyl transferase and a β1-4 galactosyl transferase capable of forming a β1-4 bond to a glucose on the acceptor molecule. Whilst it might be desirable that the exogenous glycosyl tranferases add sugars to a terminal sugar residue of the acceptor molecule, it may be adequate that the glycosyl transferases compete with an endogenous transferase, and that the acceptor molecule is a partially completed endogenous oligosaccharide or polysaccharide.

Where the shiga toxin is Stx2e the optimal oligosaccharide moiety of the receptor for a terminal chain is GalNAcβ[1-3]Galα[1-4]Galβ[1-4]Glc. The one or more transferases are selected to transfer one or more sugars selected from the terminal portion of the receptor for Stx2e. In addition to those transferases listed above, the one or more transferases are selected to include a β1-3GalNAc transferase capable of forming a β1-3 bond with a galactose residue.

The clostridial toxins might be selected from the group, tetanus toxin, botulinum toxin, *C. difficile* toxins A and B. The receptor for *C. difficile* toxin A is known to contain a Galβ[1-4]GlcNAc moiety which is believed to be all that is needed for binding. The receptor for botulinum toxin is also believed to be a sialic acid containing glycoprotein or glycolipid present on neurons.

The cholera toxins might be selected from the group comprising cholera toxin and *E. coli* heat labile enterotoxin types I and II. The receptor for cholera toxin and *E. coli* heat labile enterotoxin type I is the ganglioside G_{M1}, the structure being as follows:-

Galβ[1-4]GalNAcβ[1-4]Galβ[1-4]-ceramide
|
[2-3]NeuNAcα

The receptor mimic might be a mimic of the natural receptor for adhesins or toxins produced by micro-organism selected from a group of genera comprising the following:- *Escherichia, Salmonella, Shigella, Citrobacter, Helicobacter, Yersinia, Vibrio, Aeromonas, Campylobacter, Pseudomonas, Pasteurella, Neisseria, Haemophilus, Klebsiella, Staphylococcus, Streptococcus, Clostridium*, as well as viruses including rotavirus.

In one form the acceptor molecule is a molecule which comprises at least some sugar units, these sugar units are normally exposed to the outside of the delivery bacterium. A preferred acceptor molecule is a lipopolysacchride (LPS) molecule, and the acceptor molecule is all or a portion of the core of the lipopolysaccharide.

In the case where the two terminal sugars of the chimeric receptor binds to Stx, Stx1, Stx2, Stx2c, and Stx2d, it may be preferred that the acceptor molecule has a terminal glucose. The acceptor molecules might be the LPS core of *waaO* mutants of *Escherichia coli* R1 wherein a terminal glucose is presented in the completed acceptor molecule of the mutant concerned. Alternatively a terminal glucose need not be provided, and the choice of bacterium based on LPS core structure is wide indeed. Specific examples of core structure that are suitable as acceptor molecules include but are not limited to the following:-

Escherichia coli R1, including *waaO* mutants thereof, *Escherichia coli* K-12 and various *waa* mutants thereof, *Salmonella typhimurium* LT2, and various *waa* mutants thereof. It will be understood however that the range of molecules from which the acceptor molecule might be chosen is wide.

The acceptor molecule might be another glycolipid, glycoprotein, or other carbohydrate that is anchored in the delivery bacteria, for example, capsular polysaccharides of either gram-negative or gram-positive bacteria, or teichoic acids and lipoteichoic acids of gram-positive bacteria.

In another form the acceptor molecule is one that is normally secreted into the external milieu, such as exopolysaccharides of either gram-negative or gram-positive bacteria.

The delivery bacterium is chosen to be non-harmful when administered. It is also possible that a pathogen or an organism with potentially adverse health effects is used, and this would be administered in an attenuated form, or may be administered in a killed form. The killing of the bacterium is to be conducted under standard conditions that maintain the chimeric carbohydrate molecule, and in particular the receptor mimic intact. Examples of methods of providing killed bacteria include, but are not limited to, treatment with chemical agents such as formalin, thiomersal, or streptomycin or other bactericidal antibiotic, or exposure to heat or UV irradiation. Bacterial ghosts generated by induction of a bacteriophage lysis protein may also be a suitable delivery vehicle for receptor mimics, as would liposomes incorporating the chimeric LPS. In the alternative purified membrane vesicles (MVs) might be used. MVs are naturally relaeased by gram negative bacteria and might be described as blebs of the outer membrance. In all these cases, this would require that a sufficient dose be given to permit passage of sufficient ligand binding capacity to the site of infection or potential infection.

Preferably however the delivery bacterium is utilised in a live form so that it can multiply *in vivo* at least to a limited extent, thereby producing more receptor mimics, so that smaller doses can be effective.

There may be inherent advantages in having the receptor formed by bacteria *in situ*, including cost, capacity of the delivery bacterium to undergo multiplication in the gut, expression of a high density of receptor mimics on the cell surface, and display of the receptor mimic in an appropriate conformation.

The stomach of an individual presents a considerable barrier to the introduction of bacteria and acid labile macromolecules. It might be desirable to provide for some acid resistance. For example a particle might be delivered that has a protective coating, such as those that are commonly used in the delivery of pharmaceuticals, which can then be selectively released either in the small intestine, large intestine or both. Such coatings and capsules are well known. An alternative approach might be to make the delivery bacteria more acid tolerant, for example the delivery bacteria could have a constitutive acid-tolerance response. It might also be desired to grow the delivery bacteria in media that enhance their capacity to

pass the acid barrier of the stomach; for example the culture may be grown under low pH concentrations to induce an acid tolerance response.

Additionally it might be desired to provide for some resistance to anti microbial activity that is presented by the resident microflora. Thus for example the delivery bacteria could be modified or chosen to be resistant to the major families of colicins (for example Col E1, E2 and E3) by the introduction of the *btuB* mutation.

The choice of delivery bacterium is wide insofar as what is required is that the organism is capable of expressing the chimeric carbohydrate molecule being delivered to the gut. The delivery mode might be in a protected environment such as by being coated or inside a capsule, and thus the organism need not necessarily be acid resistant. It is also possble that the delivery bacterium need not survive in the gut. All that is required is that the receptor mimic be exposed in sufficient quantities to adsorb toxin or the pathogenic organism. It is preferred however that the organism does survive and grow and therefore presents an increasing level of the chimeric carbohydrate for adsorption, accordingly it is desired that the organism is resistant to conditions found in the gut and thus is an enteric organism.

The delivery bacterium might be selected from the genuses Escherichia and Salmonella, and more preferably *Escherichia coli* and *Salmonella enterica* sv typhimurium. However, certain other bacterial genera may be convenient to use, notably those such as Acidophilus, Lactobacillus, Lactococcus or Bifidobacterium, which being food bacteria, are known to be safe to administer orally to humans, and are also capable of survival in the gut. These later organisms have, in common with *Escherichia* and *Salmonella* been relatively well characterised.

To be more effective it is desired that the delivery bacterium properly exposes the receptor mimic in as effective a manner as possible. Accordingly it is desired that the delivery bacterium does not produce or has a limited capacity to produce any of the following: O-antigen, a slime layer, capsule or exopolysaccharide, where such carbohydrates are not the carrier of the receptor mimic. These molecules might otherwise mask the receptor mimic.

It is also desirable that the bacterium expressing the receptor mimic colonizes the gastrointestinal tract for only a limited period of time, so as not to perturb normal receptor-ligand interactions, or disturb the state of immune tolerance which prevents elicitation of potentially deleterious anti-receptor antibodies. Incorporation of an inducible suicide gene

may help eliminate the recombinant bacterium from the host when its presence is no longer necessary. An example of such as system is the stochastic lethal containment system described by Klemm *et al* (42)

The elucidation of the nature of the oligosaccharide receptors is an ongoing endeavour, and more receptors for toxins and adhesins are being defined as time goes on. Selecting appropriate carbohydrate structures can be achieved by searching through a database of known carbohydrate structures, such as Carbbank, which is available over the internet; CDrom versions are also available from NBRF, National Biomedical Research Foundation, 3900 Reservoir Road, NW, Washington DC 20007 USA. Selecting appropriate nucleic acid sequences for expression of the desired glycosyl transferases can be achieved by searching through a database of genes encoding glycosyl transferases available over the internet, such as CAZy. This database is administered by AFMB-CNRS a contact being at 31 Chemin Joseph Aiguier F-13402 Marseille Cedex 20 (France). An alternative is to search for structures in the Chemical Abstracts. The search concerned will identify the sugar specificity of the transferase, the sugar to which it binds, the nature of the bond, and the overall nature of the acceptor molecule. Thus where the acceptor molecule is a LPS then a transferase specific for LPS will be preferred. The gene encoding the transferase of interest can be either made synthetically or alternatively isolated from an appropriate organism and incorporated into an expression vector.

It is envisaged that the glycosyl transferase will be a naturally occurring glycosyl transferase, however, the naturally occurring transferase may need to be modified, to change the specificity or to stabilise a gene that might be subject to phase variation during normal cellular processes such as replication.

For Stx, Stx1, Stx2, Stx2c, and Stx2d receptors, the N-terminal sugar transferase gene may be selected from the group comprising *lgtC* of *N. meningitidis* or *N. gonorrhoeae* and the penultimate terminal sugar transferase gene may be selected from the group comprising *lgtE* of *N meningitidis* or *N. gonorrhoeae*; *Haemophilus influenzae* strain Rd is also known to contain genes encoding enzymes with analogous functions.

In the case of K88ad adhesin its receptor includes the lactoneotetraose series of glycolipids. These carbohydrates are produced by *N meningitidis*, *N. gonorrhoeae*, and some strains of *Haemophilus influenzae* as one of their variable LOS components, and genetic material from these strains could be used to construct a recombinant receptor mimic.

DNA encoding the exogenous genes may be carried on a non-integrated vector such as a plasmid, selected to be stable within the delivery bacterium. Such vectors are known to those skilled in the art. One benefit in having the DNA in a non-integrated form is that a high copy number of the encoding DNA can mean that where competitive addition of sugars to an intermediate acceptor molecule is required, the enzyme encoded by the high copy number gene can prevail. An alternative is to have the exogenous gene or genes incorporated into the bacterial chromosome. This tends to provide a greater measure of stability.

The predominant focus of the descriptions in this specification are the clearing and/or neutralization of enteric organisms or toxins, however broader aspects of the invention are not so restricted and it may be desired to reduce the number of pathogenic organisms from other environments such as the oral cavity, the urogenital tract, or external environments such as waterways.

DETAILED DESCRIPTION OF THE INVENTION.

For a better understanding the invention will now be described with reference to the examples.

Background

Shiga toxin- (Stx-) producing strains of *Escherichia coli* (STEC) are important causes of diarrhoea and haemorrhagic colitis (HC) in humans. This can lead to potentially fatal systemic sequelae, such as haemolytic uraemic syndrome (HUS) which is the leading cause of acute renal failure in children (1, 2, 3, 4). Certain other *Enterobacteriaceae* are also known to produce Stx and cause serious gastrointestinal disease in humans. The most notable of these is *Shigella dysenteriae* type 1, the causative agent of bacillary dysentery, which is frequently associated with Stx-induced systemic sequelae, including HUS (1). Indeed, it is the principal cause of HUS in parts of Africa and Asia (4). Stx-producing *Citrobacter freundii* has also been shown to cause diarrhea and HUS in humans, including one outbreak in a German child-care centre (4).

The mortality rate for HUS is 5-10%; other acute complications include stroke, diabetes mellitus, and necrotising colitis necessitating colectomy. The Centers for Disease Control and Prevention (Atlanta, GA) has estimated that the annual cost for acute care of patients with STEC disease in the USA is in the range of $1-2 billion, with approximately 500

deaths each year (3). In addition, up to a third of survivors sustain permanent renal impairment and may eventually require transplantation (2). Estimates of the on-going cost of management of these long-term complications are not available.

STEC are commonly found in the intestines of livestock, and human infections usually result from consumption of contaminated meat or dairy products; fruit and salad vegetables contaminated with manure, and contaminated drinking or swimming water are also common STEC vehicles. In addition, approximately 20% of all cases of STEC disease are believed to result from person to person transmission (3, 6). STEC belonging to over 100 O:H serotypes have been associated with human disease. However, those belonging to serogroup O157 (particularly O157:H7) are the most prevalent causes of HUS and account for the majority of the major food-bourne outbreaks in the United States, Europe and Britain (2, 3). It is likely, however, that the epidemiological data on the overall incidence of STEC disease and serotype prevalence has been skewed by underdetection of cases caused by non-O157 STEC, which are much more difficult to detect (3, 4). In recent years there have been a number of large outbreaks of STEC disease in North America, the UK, Europe, Japan and Australia. Such outbreaks have the potential to overwhelm acute care facilities, even in developed countries with sophisticated health-care systems. The largest (Sakai, Japan, May-June 1996) involved over 8,000 cases of HC (600 requiring hospitalization) and 107 cases of HUS. Another outbreak involving over 500 people and 20 deaths occurred in Scotland in December 1996.

Stx is a compound toxin, consisting of an enzymatically active A subunit (an RNA-*N*-glycosidase), which inhibits eukaryotic protein synthesis, and a pentameric B subunit responsible for binding to glycolipid receptors in target cell membranes (2). Two major classes of Stx (Stx1 and Stx2) have been distinguished, both by serological methods, as well as by DNA sequence analysis. Individual STEC strains may produce toxins belonging to either or both of the two major Stx classes. However, substantial variation in amino acid sequence occurs within both the Stx1 and Stx2 groups (7, 8, 9, 10,11,12). Within the Stx2 class, several subtypes have been distinguished on the basis of differences in biological properties. All Stx types associated with human disease recognize the same glycolipid receptor, globotriaosyl ceramide (Gb_3), which has the structure Galα[1-4]Galβ[1-4]Glc-ceramide (13). Vero (African green monkey kidney) cells express large amounts of Gb_3 on their surface, consequently, this cell line is highly susceptible to Stx, and Vero cytotoxicity is the generally recognised standard assay for Stx activity. However, one particular subgroup of Stx2 variants, designated Stx2c, share specific B subunit amino

acid differences with respect to classical Stx2 (Asp_{16}-Asn and Asp_{24}-Ala), which correlate with a somewhat reduced binding affinity for the receptor Gb_3 and reduced *in vitro* cytotoxicity for HeLa cells (14). A separate subgroup, designated Stx2d, is distinguished from other Stx toxins by increased cytotoxicity for Vero cells after incubation in the presence of either mouse proximal small intestinal mucus or human colonic mucus. Activation appears to be a function of the A subunit, because the B subunit is identical to Stx2c, which was not activatable. Activatable Stx2d toxins examined to date share two A subunit amino acid differences with respect to Stx2c (Ser_{291} and Glu_{297}), although these alone may not necessarily be sufficient for activation, as they are also found in some Stx2 subtypes which are not activatable (15). A final major Stx2 subtype is Stx2e, which is produced by STEC associated with piglet oedema disease. This is a serious, frequently fatal illness affecting piglets at the time of weaning, and is characterized by neurological symptoms including ataxia, convulsions and paralysis; oedema is typically present in the eyelids, brain, stomach, intestine and mesentery of the colon (16). It is associated with particular STEC serotypes (most commonly O138:K81, O139:K82 and O141:K85, which are not associated with human disease. These particular STEC strains also cause post-weaning diarrhoea in piglets. Stx2e has a different glycolipid receptor specificity from other members of the Stx family, recognising globotetraosyl ceramide (Gb_4; GalNAcβ[1-3]Galα[1-4]Galβ[1-4]Glc-ceramide) preferentially over Gb_3 (17). Two amino acid differences in the Stx2e B subunit (Gln_{64} and Lys_{66}) are critical for this altered specificity, which impacts on the tissue tropism of the toxin, thereby accounting for the distinctive clinical presentation of oedema disease (18).

The pathological features seen in severe human STEC disease (HC and HUS) are directly attributable to the Stx toxins, which are essential for virulence. Pathogenesis of disease initially involves colonization of the gut by the STEC; the bacteria do not invade the gut epithelium, but locally produced Stx is absorbed into the circulation, and the toxin then targets specific tissues in accordance with their Gb_3 content. In humans Gb_3 is found in highest concentrations in renal tissue, and in microvascular endothelial cells (particularly in the kidneys, gut, pancreas and brain), thereby accounting for the distinct clinical and pathological features of HUS (microangiopathic haemolytic anaemia, thrombocytopoenia and renal failure).

There is increasing evidence that STEC strains vary in their capacity to cause serious disease in humans, and that this, at least in part, is a function of the type and/or amount of Stx produced. Indeed, up to 1000-fold differences in the cytotoxicities of various STEC

isolates from humans have been reported. Moreover, patients infected with STEC producing Stx2 are more likely to develop serious complications such as HUS than those infected with STEC producing Stx1 (19,20). The link between Stx2 production and HUS may be a direct consequence of increased *in vivo* toxicity of Stx2. Indeed, human renal microvascular endothelial cells have been shown to be far more susceptible to the cytotoxic action of Stx2 than Stx1 (21). This is consistent with studies employing a streptomycin-treated mouse model of toxin-induced renal tubular damage. Oral challenge with *E. coli* K-12 carrying cloned *Stx2* genes, but not *Stx1* genes was capable of inducing fatal tubular damage (22).

The availability of rapid and sensitive methods for diagnosis of STEC infection early in the course of disease has created a window of opportunity for therapeutic intervention. Indeed, during two outbreaks which have occurred in Adelaide we diagnosed STEC infection in patients by PCR almost a week before symptoms of HUS became apparent. The increased awareness that occurs during major outbreaks is likely to result in more patients presenting during the early (diarrhoeal) stage, and when a source of infection has been identified and publicised, persons exposed to the contaminated product may come forward before symptoms appear. An opportunity also exists to treat close contacts of persons with proven or suspected STEC infection (e.g. family members, children in child-care centres, school classmates, etc.), to prevent them from developing serious disease. Antibiotic therapy is contraindicated for STEC infection because of the risk of increasing free Stx in the gut lumen through release of cell-associated toxin and induction of toxin gene expression. There are also concerns that antibiotic therapy might disturb gut flora and result in overgrowth by the STEC. Administration of antimotility agents is also contraindicated (2,4).

During the early stages of human infections, STEC may colonize the gut at high levels (>90% of aerobic flora), exposing the host to sustained high concentrations of Stx and increasing the likelihood of systemic complications. However, as disease progresses, the numbers of STEC decrease markedly, and may even be undetectable in patients who have already progressed to HUS. Western blot analysis using convalescent sera from HUS patients suggests that the elimination of STEC from the gut during the latter stages of HUS is probably a consequence of local immune responses to STEC surface antigens (23). Clearly, in cases of natural STEC infection, the immune response occurs too slowly to prevent Stx-induced complications. Thus, *in vivo* binding or neutralization of Stx is a

potentially important therapeutic strategy. Substances capable of binding Stx in the gut might also play a role as an adjunct to antibiotic therapy.

All Stx types affecting humans recognise the same glycolipid receptor (Gb_3), and at least one strategy exploiting this interaction has been developed. This agent, called Synsorb-Pk, consists of chemically synthesised Galα[1-4]Galβ[1-4]Glc- (the trisaccharide component of Gb_3) covalently linked via an 8 carbon spacer to silica particles derived from diatomaceous earth. Synsorb-Pk is capable of binding and neutralizing Stx1 and Stx2 in STEC culture extracts, and in faeces from patients with HC and HUS, although binding of Stx2-related toxins is less efficient than for Stx1 (24,25). 1 mg of Synsorb-Pk has been shown to be capable of binding 93% of a 0.5 ng aliquot of radioiodinated Stx1 (24). Other *in vitro* studies using purified toxins indicate that the saturation binding capacity of 1 mg of Synsorb-Pk is approximately 5 ng of purified Stx1 or Stx2 (26). In this latter study coincubation experiments indicated that 1 mg of Synsorb-Pk could protect 50% of cells in tissue culture from 2 ng of Stx1 or 0.4 ng of Stx2. A phase I clinical trial did not detect any adverse effects associated with oral administration, and Synsorb-Pk retained its Stx-binding capacity after passage through the human gastrointestinal tract (27). Results of a randomized, double-blind trial vs placebo in children with STEC diarrhoea indicated that oral administration of approximately 500 mg Synsorb-Pk per kg per day reduced the relative risk of progression to HUS by approximately 50%, but only if administered within 3 days of onset of disease (25).

EXAMPLE

This example, the invention resides in construction of a harmless recombinant delivery bacterium capable of incorporating the trisaccharide Galα[1-4]Galβ[1-4]Glc- into the outer core region of its lipopolysaccharide, such that a mimic of the natural host receptor for the toxin is displayed on the bacterial surface. This delivery bacterium, either live or killed, is capable of binding all Stx types associated with human disease. Surprisingly, this bacterium binds Stx2, Stx2c, and Stx2d toxins at least as effectively as it does Stx1. This is a very important property, since the Stx2-related toxins have greater toxicity for human renal microvascular endothelial cells than Stx1, and strains producing Stx2 are more frequently associated with HUS than those producing only Stx1, as described above. Moreover, this finding was unexpected, given that the synthetic product Synsorb-Pk has a lower binding affinity for Stx2-related toxins than it has for Stx1 (see above). In addition, the recombinant delivery bacterium is capable of binding the oedema disease-associated toxin Stx2e, albeit less efficiently than the other Stx types.

Procedure for construction of recombinant bacteria expressing the trisaccharide Galα[1-4]Galβ[1-4]Glc- on their surface.

In order to locate a source of bacterial genes encoding biosynthesis of the trisaccharide component of the receptor for Stx, we conducted a search of carbohydrate structure databases. This revealed that Galα[1-4]Galβ[1-4]Glc- is found in the outer core region of the lipooligosaccharides (LOS) of *Neisseria meningitidis* (28) and *N. gonorrhoeae* (29), as well as in the LPS of certain strains of *Haemophilus influenzae* (30). LOS are the major glycolipids expressed on the surface of these bacteria, and are analogous to the LPS of other Gram-negative bacterial genera such as *Escherichia* and *Salmonella*. LOS and LPS have many structural features in common. Lipid A is the common hydrophobic moiety, and this forms the outer leaflet of the outer membrane. This is linked to the inner core oligosaccharide, which consists largely of heptose and KDO (3-deoxy-D-manno-oct-2-ulosonic acid). The general structural features of lipid A and the inner core oligosaccharide are highly conserved amongst diverse bacterial species, as they are important for outer membrane stability (31,32). The outer region of the core oligosaccharide comprises hexoses, which are linked to the inner core by a variety of highly specific glycosyl transferases. Thus, this region varies in structure from species to species and even within a given bacterial strain (see below). In Enterobacteriaceae, an antigenic repeat-structure O-polysaccharide is attached to the distal end of the outer core oligosaccharide to form high molecular weight LPS. However, in genera such as *Neisseria*, this does not occur, resulting in a much lower molecular weight LOS (31,32). Pathogenic species of *Neisseria* have also been shown to sialylate their LOS, an important virulence trait, as this confers a high degree of resistance to the bactericidal activity of serum (29,31).

Another important property of *N. gonorrhoeae* and *N. meningitidis* is the capacity to undergo phase variation which alters the structure (and hence the antigenicity) of the outer core oligosaccharide, thereby avoiding host immune responses. In both species, one of the alternative LOS immunotypes has an outer core consisting of Galα[1-4]Galβ[1-4]Glc- (referred to as immunotype L1). Phase variation occurs spontaneously and at such high frequency that in any given culture, cells expressing LOS with different outer core oligosaccharide immunotypes will be present. This, as well as the fact that they are pathogens, precludes use of these *Neisseria* sp. as *in vivo* toxin-binding agents. Genetic characterization of the region of the *N. gonorrhoeae* chromosome encoding outer core biosynthesis has provided a molecular explanation for the LOS phase variation phenomenon (33,34). This region contains five glycosyl transferase genes, *lgtA*, *lgtB*,

lgtC, *lgtD* and *lgtE*, arranged as an operon. Three of these genes (*lgtA*, *lgtC* and *lgtD*) contain poly-G tracts within the respective open reading frame, which renders them highly susceptible to slipped strand mispairing during replication. Such slippage results in frame shift mutations and concomitant premature termination of translation of the respective specific glycosyl tranferase (34). Thus, the actual outer core oligosaccharide structure, and hence immunotype, will depend on which of the *lgt* genes are encoding functional enzymes at a given point in time. The exact specificity of the five gonococcal glycosyl transferases has been determined by mutational analysis (34). From this we have deduced that expression of the L1 immunotype LOS requires functional *lgtE* and *lgtC* genes, which encode the transferases resposible for linking the α-galactosyl and β-galactosyl residues onto the glucose residue attached to the distal end of the inner core oligosaccharide. For this to happen in *Neisseria*, *lgtA* must be non-functional, as this gene encodes a highly active *N*-acetylglucosamine transferase which adds Glc*N*Ac to the outer core in lieu of the terminal α-Gal. The products of *lgtB* and *lgtD*, on the other hand, do not appear to interfere with synthesis of immunotype L1, presumably because these transferases have absolute specificity for acceptor oligosaccharides containing Glc*N*Ac (34).

Sequence data for the entire *lgt* region from both *N. gonorrhoeae* and *N. meningitidis* are available on the GenBank database (accession numbers U14554 and U65788, respectively). This was used to design the following oligonucleotide primers to direct amplification of *lgtC* and *lgtE* genes:

Primer (underlined)	**Sequence (5'-3')**	**Restriction site inserted**
LGTCF	GAACAGGAATTCGGCAAGATTATTGTGCC	*Eco*RI
LGTCR	TACGTCGGATCCCGTCTGAAGGCTTCAGAC	*Bam*HI
LGTEF	GCCCTTGGATCCACCGCAGCTATTGAAACC	*Bam*HI
LGTER	CCATTTAAGCTTTTAATCCCCTATATTTTACAC	*Hin*dIII

The *lgtC* and *lgtE* genes were PCR amplified using primer pairs LGTCF/LGTCR and LGTEF/LGTER, respectively, with *N. meningitidis* and *N. gonorrhoeae* DNA as template, respectively (the *lgtC* and *lgtE* genes from the two species are approximately 95%

identical). These PCR products were cloned into the vector pK184 (35) after digestion of both vector and PCR product with *Eco*RI/*Bam*HI or *Bam*HI/*Hin*dIII, respectively, and transformed into *E. coli* K-12. Since the *lgtC* gene is one of those with a poly-G tract, it was necessary to mutate this region to stabilize expression of the encoded transferase. The DNA sequence of *N. meningitidis lgtC* from nt 157-171 of the open reading frame is CGGGGGGGGGGGGGT, which encodes the amino acid sequence Arg-Gly-Gly-Gly-Gly. This region of the *lgtC* gene cloned in pK184 was mutated to CGTGGCGGTGGCGGT by overlap extension PCR. This involved separate PCR amplification of overlapping 5' and 3' portions of the cloned *lgtC* gene. The 5' portion was amplified using the universal M13 reverse sequencing primer and another with the sequence ATATTACCGCCACCGCCACGCAAATTGGCGGC, whereas the 3' portion was amplified using the universal M13 forward sequencing primer and another with the sequence AATTTGCGTGGCGGTGGCGGTAATATCCGCTT. The two PCR products were then purified, aliquots were mixed, and full length *lgtC* with the desired modifications was amplified by PCR using the M13 forward and reverse primers. The PCR product was digested with *Eco*RI/*Bam*HI and once again cloned into similarly digested pK184, and subjected to sequence analysis to confirm mutagenesis of the poly G tract. This eliminated the possibility of slipped strand mispairing without affecting the amino acid sequence of the encoded protein. The mutated *lgtC* gene was then excised from the pK184 construct with *Eco*RI/*Bam*HI and cloned into the compatible restriction sites in the pK184 derivative containing *lgtE*. This places the *lgtC* and *lgtE* genes in tandem in pK184, in the same orientation as the vector *lac* promoter. The complete DNA sequence of the *lgtCE* insert in pK184 is shown in Fig. 1.

The recombinant pK184:*lgtCE* plasmid was then transformed into a suitable *E. coli* host. In the first instance we used a derivative of *E. coli* R1 (designated CWG308) which has a non-polar insertion mutation in the *waaO* gene, resulting in expression of an LPS core consisting of just the inner core plus Glc linked to the terminal heptose residue (36). This structure is very similar to the natural substrate for the galactosyl transferase LgtE, and so CWG308 is an appropriate host for expression of *lgtCE*. A derivative of *E. coli* K-12 with mutations in *waaO* and *waaB* is also a suitable host for expression of *lgtCE*, as it has the same lipopolysaccharide core structure as CWG308. This host has an additional advantage in that it has been proven to be safe for oral administration to humans in very high doses (37). Extensive studies carried out in the early 1980s demonstrated that although it is capable of growth in the human gut, it can not establish long-term, high level colonization, as it lacks adhesins found in pathogenic strains of *E. coli* (37).

Transformation of CWG308 with pK184/*lgtCE* resulted in synthesis of LPS with an outer core oligosaccharide containing a terminal Galα[1-4]Galβ[1-4]Glc- epitope, as judged by reactivity on dot-immunoblot with a monoclonal antibody specific for the *N. meningitidis* L1 immunotype. Moreover, CWG308:pK184/*lgtCE* is capable of directly binding and neutralizing Stx, as detailed below.

Procedure for testing the capacity of recombinant bacteria to neutralise Stx
CWG308 and CWG308:pK184/*lgtCE* were grown overnight at 37°C in LB broth (supplemented with 25 μg/ml kanamycin in the case of CWG308:pK184/*lgtCE*), harvested by centrifugation, washed and resuspended in phosphate-buffered saline (PBS) at a density of approximately 1 X 10^9 CFU/ml (equivalent to approximately 2mg dry weight of cells per ml). In the first instance, French pressure cell (FPC) lysates of fresh overnight LB broth cultures of the following *E. coli* strains were used as a source of Stx.

Strain	**Description**	**Ref.**
EDL933	Wild type O157:H7 STEC producing Stx1 and Stx2	(38)
JM109:pJCP521	*E. coli* JM109 with Stx_{2c} cloned in pBluescript	(11)
JM109:pJCP525	*E. coli* JM109 with Stx_1 cloned in pBluescript	(9)
JM109:pJCP539	*E. coli* JM109 with Stx_2 cloned in pBluescript	(39)
JM109:pJCP542	*E. coli* JM109 with Stx_{2d} cloned in pBluescript	(8)
128/12	Wild type piglet oedema disease STEC producing Stx2e	

FPC lysates of each of the above cultures were filter-sterilized and 0.5 ml aliquots were incubated with 1 ml of CWG308 or CWG308:pK184/*lgtCE* suspension, or PBS, for 1 hour at 37°C with gentle agitation. The mixtures were then centrifuged and filter-sterilized. Twelve serial 2-fold dilutions were prepared in tissue culture medium (Dulbecco's Modified Eagles Medium buffered with 20 mM HEPES, and supplemented with 2 mM L-glutamine, 50 IU/ml penicillin and 50 μg/ml streptomycin), commencing at a dilution of 1:20. Fifty μl of each dilution was transferred onto washed Vero cell monolayers in 96-well tissue culture trays, and after 30 min incubation at 37°C, a further 150 μl of culture medium was added to each well. Cells were examined microscopically after 72 hours incubation at 37°C, and scored for cytotoxicity. The endpoint Stx titre was defined as the reciprocal of the highest dilution which still resulted in detectable cytotoxicity. Results for the various Stx extracts are shown below.

			% Stx neutralized by:	
Stx source	**Stx type**	**Stx titre**	**CWG308**	**CWG308:pK184/*lgtCE***
EDL933	Stx1 & Stx2	40960	0	99.6
JM109:pJCP521	Stx2c	10240	0	99.8
JM109:pJCP525	Stx1	>40960	0	99.6
JM109:pJCP539	Stx2	10240	0	98.4
JM109:pJCP542	Stx2d	1280	0	> 99.2
128/12	Stx2e	1280	0	87.5

The capacity of killed CWG308:pK184/*lgtCE* cells to bind and neutralize Stx was also examined. Cell suspensions were killed by heating at 65°C for 3 hours, or by treatment with 1% formaldehyde for 16 hours at 4°C. Capacity to neutralize cytotoxicity in FPC extracts containing Stx1 or Stx2c was then compared with that for live CWG308:pK184/*lgtCE* cells, under the standard conditions described above. The Stx titres for the Stx1 and Stx2c extracts used in this experiment were 40960 in both cases. Heat-killed CWG308:pK184/*lgtCE* neutralized 93.7% of the Stx1 and 96.8% of the Stx2c. Formaldehyde-killed CWG308:pK184/*lgtCE* neutralized 99.6% of the Stx1 and 99.2% of the Stx2c. Live CWG308:pK184/*lgtCE* cells neutralized 99.2% of the Stx1 and 99.6% of the Stx2c. Thus, heat-treatment slightly reduces the capacity of CWG308:pK184/*lgtCE* cells to bind and neutralize Stx, but formaldehyde-killed CWG308:pK184/*lgtCE* cells are as effective as live cells. Whilst it is preferable to use live CWG308:pK184/*lgtCE* in the clinical setting because of its capacity to multiply in the gut, thereby increasing the number of cells capable of binding Stx, formaldehyde-killed cells could be used in circumstances where administration of live cells is contraindicated (e.g. immunocompromised patients).

Presence of both *lgtC* and *LgtE* genes in the recombinant plasmid was essential, as CWG308 carrying derivatives of pK184 containing either *lgtC* or *lgtE* alone did not bind Stx toxin.

Strains with mutations in genes encoding outer core glycosyl transferases such that a rough LPS comprising the inner core plus Glc linked to the terminal heptose residue is produced might be expected to be preferred hosts for expression of *lgtCE*. However, it is possible that if expression of these genes in the heterologous host is sufficient, the two encoded galactosyl transferases may compete with endogenous transferases in host strains lacking outer core mutations. This may direct biosynthesis of a modified LPS containing a Stx-binding epitope. This was examined by transforming a range of wild type and mutant *E. coli* and *Salmonella typhimurium* LT2 strains with pK184/*lgtCE* and examining the capacity to bind Stx. Experimental conditions were the same as those used for the CWG308 derivative. FPC lysates of *E. coli* JM109:pJCP525 and JM109:pJCP521 were used as a source of Stx1 and Stx2c, respectively.

	% Stx1 neutralized		**% Stx2c neutralized**	
Strain	**-pK184/*lgtCE***	**+pK184/*lgtCE***	**-pK184/*lgtCE***	**+pK184/*lgtCE***
E. coli K-12 C600	0	99.6	0	99.2
E. coli K-12 D21	0	99.2	0	98.4
E. coli K-12 D21e7	0	99.6	0	93.7
E. coli K-12 D21f1	0	98.4	0	98.4
E. coli B BL21	0	0	0	0
S. typhimurium LT2 SL3748	0	99.6	0	98.4
S. typhimurium LT2 SL3750	0	99.2	0	96.8
S. typhimurium LT2 SL3769	0	93.7	0	0

Thus, host strains suitable for expression of *lgtCE* are not limited to strains with mutations in outer core LPS synthesis. Vectors other than pK184 may also be suitable for expression of these genes, including those with higher or lower copy number, different strength promoters (either constitutive or inducible), and those which utilize alternative selection markers, e.g. alternative antibiotic resistance genes, or markers capable of complementing auxotrophic mutations, such as *thyA*$^+$. Alternatively, the *lgtC* and *lgtE* genes could be integrated into the host chromosome by allellic exchange using an appropriate suicide vector such as pCACTUS, or others known to those skilled in the art.

Procedure for measuring total Stx binding capacity of CWG308:pK184/lgtCE.
To determine the total binding capacity of CWG308:pK184/*lgtCE* cells, suspensions containing 5 X 10^8 CFU (1 mg dry weight) in PBS were incubated at 37°C for 1 hour with aliquots (ranging from 1 ng to 640 µg) of purified Stx1 and Stx2 (obtained from Toxin Technologies Inc., Florida, USA) in a final volume of 0.5 ml, and cytotoxicity was compared with that for similar aliquots of toxin incubated with CWG308.

Amount of Stx	**% Stx1 neutralized**	**% Stx2 neutralized**
1 ng	99.98	99.95
5 ng	99.98	99.95
20 ng	99.98	99.95
50 ng	99.95	99.9
100 ng	99.95	99.9
200 ng	99.95	99.9
500 ng	99.2	99.9
1 µg	99.2	99.2
2 µg	99.2	99.2
4 µg	98.4	98.4
8 µg	98.4	96.8
16 µg	98.4	96.8
32 µg	98.4	96.8
40 µg	98.4	96.8
80 µg	98.4	93.7
160 µg	87.5	87.5
320 µg	50	50
640 µg	0	0

From the above it can be seen that the saturation Stx binding capacity of 1 mg of CWG308:pK184/*lgtCE* cells is approximately 100 μg for both Stx1 and Stx2. This binding capacity is more than 10,000 times greater than that claimed for Synsorb-Pk (25,26).

Procedure for testing capacity of live CWG308:pK184/lgtCE cells to protect mice from fatal infection with STEC.

A streptomycin-treated mouse model of lethal Stx2-induced renal damage has been described previously (12,22,40). Two wild type STEC strains were used; B2F1 (which produces Stx2d), and 97MW1 (which produces Stx2). B2F1 is known to have very high virulence in this model; mice fed as few as 10 organisms succumb (41). Two groups of 8 streptomycin-treated balb/C mice were challenged with approximately 1 X 10^8 CFU of STEC B2F1; another two groups of 8 mice were challenged with STEC 97MW1. Mice were then given oral doses of approximately 4 X 10^9 CFU of either CWG308 or CWG308:pK184/*lgtCE* suspended in 60 μl of 20% sucrose, 10% $NaHCO_3$, twice per day. The numbers of STEC, as well as either CWG308 or CWG308:pK184/*lgtCE*, as appropriate, were monitored in faecal samples from each group. One day (24 hours) after challenge, faecal pellets contained approximately 10^9 CFU of the respective STEC per g. Faecal pellets from groups which received CWG308:pK184/*lgtCE* also contained approximately 10^3 CFU of this strain. For both the B2F1 and 97MW1 groups, all of the mice which received oral CWG308 died (median survival time 4 days). However, all of the mice which received CWG308:pK184/*lgtCE* survived and were alive and well two weeks after challenge. This difference in survival rate (8/8 vs 0/8) is highly significant ($P << 0.005$; Fisher exact test) and demonstrates unequivocally that oral administration of CWG308:pK184/*lgtCE* is capable of preventing the fatal systemic complications of STEC disease.

REFERENCES

1. O'Brien & Holmes (1987). *Microbiol Rev* **51**:206-20.
2. Karmali (1989). *Clin Microbiol Rev* **2**:15-38.
3. Nataro & Kaper (1998). *Clin Microbiol Rev* **11**:142-201.
4. Paton & Paton (1998). *Clin. Microbiol. Rev.* **11**:450-479.
5. Keusch. (1997) Passive and active immunization against STEC and HUS. 3rd International Symposium and Workshop on Shiga Toxin (Verotoxin) -producing *Escherichia coli* Infections, Baltimore, MD, USA. June 1997.

6. Griffin. (1997) Overview of epidemiology of STEC infections in humans. 3rd International Symposium and Workshop on Shiga Toxin (Verotoxin) -producing *Escherichia coli* Infections, Baltimore, MD, USA. June 1997.
7. Gannon *et al.* (1990). *J Gen Microbiol.* **136**:1125-1135.
8. Ito *et al.* (1990). *Microb. Pathogen.* **8**: 47-60.
9. Paton *et al* (1995). *Gene.* **153**:71-74.
10. Paton *et al.* (1992). *Microb. Pathog.* **13**:225-236.
11. Paton *et al.* (1993). *Microb. Pathog.* **15**:77-82.
12. Paton *et al* (1995). *Infect. Immun.* **63**:2450-2458.
13. Lingwood (1996). *Trends Microbiol.* **4**:147-153.
14. Lindgren *et al* (1994). *Infect. Immun.* **62**:623-631.
15. Melton-Celsa *et al.* (1996). *Infect. Immun.* **64**:1569-1576.
16. Imberechts *et al* (1992). *Vet. Microbiol.* **31**:221-233.
17. DeGrandis *et al.* (1989). *J. Biol. Chem.* **264**:12520-12525.
18. Tyrrell *et al* (1992). *Proc. Natl. Acad. Sci. U. S. A.* **89**:524-528.
19. Kleanthous *et al.* (1990). *Arch. Dis. Child.* **65**: 722-727.
20. Ostroff *et al.* (1989). *J. Infect. Dis.* **160**: 994-998.
21. Louise & Obrig (1995). *J. Infect. Dis.* **172**:1397-1401
22. Wadolkowski *et al.* (1990). *Infect. Immun.* **58**: 3959-65.
23. Voss *et al* (1998). *Infect. Immun.* **66**:1467-1472.
24. Armstrong *et al* (1991). *J. Infect. Dis.* **164**:1160-1167.
25. US Patent number 5849714.
26. Takeda *et al* (1999). *Microbiol. Immunol.* **43**:331-337.
27. Armstrong *et al* (1995). *J. Infect. Dis.* **171**:1042-1045.
28. Wakarchuk *et al.* (1998). *Eur. J. Biochem.* **254**:626-633.
29. Mandrell & Apicella,. (1993). *Immunobiology* **187**:382-402.
30. Risberg *et al.* (1999). *Eur. J. Biochem.* **261**: 171-180.
31. Preston *et al.* (1996). *Crit. Rev. Microbiol.* **22**:139-180.
32. Heinrichs *et al.* (1998). *Mol. Microbiol.* **30**:221-232.
33. Gotschlich. (1994). *J. Exp. Med.* **180**:2181-2190.
34. Yang & Gotschlich. (1996). *J. Exp. Med.* **183**:323-327.
35. Jobling & Holmes (1990) *Nucleic Acids Res* **18**: 5315-5316.
36. Heinrichs (1998). *J. Biol. Chem.* **273**:29497-29505.
37. Levine *et al.* (1983). *J. Infect. Dis.* **148**:699-709.
38. O'Brien *et al.* (1984). *Science* **226**:694-696.
39. Paton *et al.* (1999). *J. Clin. Microbiol.* In press.

40. Wadolkowski *et al.* (1990). *Infect. Immun.* **58**:2438-2445.
41. Lindgren *et al.* (1993). *Infect. Immun.* **61**:3832-3842.
42. Klemm et al (1995). *Appl Environ Microbiol* **61**:481-486
43. Grange et al (1999). *Infect Immun* **67**: 165-172

Fig. 1. Nucleotide sequence of *lgtCE* insert in pK184

*Eco*RI *lgtC* start

GAATTCGGCAAGATCATTGTGCCTTTCCAATAAAAGGAGAAAAGATGGACATC
GTATTTGCGGCAGACGACAACTATGCCGCCTATCTTTGCGTTGCGGCAAAAAG
CGTGGAAGCGGCCCATCCCGATACGGAAATCAGGTTCCACGTCCTCGATGCCG
GCATCAGTGAGGCAAACCGGGCGGCGGTTGCCGCCAATTTGCGTGGCGGTGG
CGGTAATATCCGCTTTATAGACGTAAACCCCGAAGATTTCGCCGGCTTCCCCTT
AAACATCAGGCACATTTCCATTACGACTTATGCCCGCCTGAAATTGGGCGAATA
CATTGCCGATTGCGATAAAGTCCTGTATCTGGATATAGACGTATTGGTCAGGGA
CAGCCTGACGCCCTTATGGGATACCGATTTGGGCGATAACTGGCTTGGCGCGT
GCATTGATTTATTTGTCGAAAGGCAGGAAGGCTACAAACAAAAAATCGGTATG
GCGGACGGCGAATATTATTTCAATGCCGGCGTATTGCTGATCAACCTGAAAAA
GTGGCGGCGGCACGATATTTTCAAAATGTCCTGCGAATGGGTGGAACAATACA
AGGACGTGATGCAATATCAGGATCAGGACATTTTGAACGGGCTGTTTAAAGGC
GGGGTGTGTTATGCGAACAGCCGTTTCAACTTTATGCCGACCAATTATGCCTTT
ATGGCGAACTGGTTTGCGTCCCGCCATACCGACCCGCTTTACCGCGACCGTAC
CAATACGGTGATGCCCGTCGCCGTCAGCCATTATTGCGGCTCGGCAAAGCCGT
GGCACAGGGACTGCACCGCGTGGGGTGCGGAACGTTTCACAGAGTTGGGCGG
CAGCCTGACGACCGTTCCCGAAGAATGGCGCGGCAAACTTGCCGTCCCGCACC
GTATGTTTTCGACAAAGCGTATGCTTCAAAGATGG

lgtC stop

CGCAGAAAGCTGTCTGCCAGATTCTTACGCAAGATTTATTGACGGGGCAGGCC
GTCTGAAGCCTTCA

*Bam*HI *lgtE* start

GACGGGATCCACCGCAGCTATTGAAACCGAACAGGATAAATCATGCAAAACCA
CGTTATCAGCTTGGCTTCCGCCGCAGAGCGCAGGGCGCACATTGCCGATACCT
TCGGCAGTCGCGGCATCCCGTTCCAGTTTTTCGACGCACTGATGCCGTCTGAAA
GGCTGGAACAGGCGATGGCGGAACTCGTCCCCGGCTTGTCGGCGCACCCCTA
TTTGAGCGGAGTGGAAAAAGCCTGCTTTATGAGCCACGCCGTATTGTGGGAAC
AGGCGTTGGATGAAGGTCTGCCGTATATCGCCGTATTTGAGGACGACGTTTTA
CTCGGCGAAGGCGCGGAGCAGTTCCTTGCCGAAGATACTTGGTTGGAAGAGC
GTTTTGACAAGGATTCCGCCTTTATCGTCCGTTTGGAAACGATGTTTGCGAAAG
TTATTGTCAGACCGGATAAAGTCCTGAATTATGAAAACCGGTCATTTCCTTTGCT
GGAGAGCGAACATTGTGGGACGGCTGGCTATATCATTTCGCGTGAGGCGATGC
GGTTTTTCTTGGACAGGTTTGCCGTTTTGCCGCCAGAGCGGATTAAAGCGGTA
GATTTGATGATGTTTACTTATTTCTTTGATAAGGAGGGGATGCCTGTTTATCAGG
TTAGTCCCGCCTTATGTACCCAAGAATTGCATTATGCCAAGTTTCTCAGTCAAAA
CAGTATGTTGGGTAGCGATTTGGAAAAAGATAGGGAACAAGGAAGAAGACACC
GCCGTTCGTTGAAGGTGATGTTTGACTTGAAGCGTGCTTTGGGTAAATTCGGTA
GGGAAAAGAAGAAAAGAATGGAGCGTCAAAGGCAGGCGGAGCTTGAGAAAGT
TTACGGCAGGCGGGTCA

lgtE stop *Hind*III

TATTGTTCAAATAGTTTGTGTAAAATATAGGGGATTAAAAGCTT

Mutated nucleotides in the former poly G tract in *lgtC* are shown in bold type and are underlined.

Dated this 10th day of September 1999

WOMEN'S AND CHILDREN'S HOSPITAL
and LUMINIS PTY LTD
By their Patent Attorneys,
A. P. T. Patent and Trade Mark Attorneys